Mobilization of Stem Cell With Granulocyte-Colony Stimulating Factor Promotes Recovery After Traumatic Brain Injury in Rat

Mohsen Marzban[1], Mehrdad Bakhtiary[1], Mehdi Mehdizadeh[1], Mohamad Taghi Joghataei[1], Samideh Khoei[2], Vahid Pirhajati Mahabadi[1], Asghar Ebrahimi[3]

1. Department of Anatomy, Iran university of Medical Sciences, Tehran, Iran
2. Department of Biophysics and Medical Physics, Iran University of Medical sciences, Tehran, Iran
3. Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milano, Italy

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ABSTRACT

Introduction: This study was designed to investigate the effects of granulocyte colony-stimulating factor (G-CSF) administration in rats for 6 weeks after traumatic brain injury (TBI).

Methods: Adult male Wistar rats (n = 30) were injured with controlled cortical impact device and divided into four groups. The treatment groups (n = 10 each) were injected subcutaneously with recombinant human G-CSF. Vehicle group (n=10) received phosphate buffered saline (PBS) and only Brdu intraperitoneally. Bromodeoxyuridine (BrdU) was used for mitotic labeling. Experimental rats were injected intraperitoneally with BrdU. Rats were killed at 6th week after traumatic brain injury. Neurological functional evaluation of animals was performed before and after injury using neurological severity scores (NSS). Animals were sacrificed 42 days after TBI and brain sections were stained using Brdu immunohistochemistry.

Results: Statistically significant improvement in functional outcome was observed in treatment groups when compared with control ($p<0.01$). This benefit was visible 7 days after TBI and persisted until 42 days (end of trial). Histological analysis showed that Brdu cell positive was more in the lesion boundary zone at treatment animal group than all injected animals.

Discussion: We believe that G-CSF therapeutic protocol reported here represents an attractive strategy for the development of a clinically significant noninvasive traumatic brain injury therapy.

1. Introduction

CNS injury as traumatic brain injury and stroke are the leading causes of death and disability worldwide with no effective treatment that enhances traumatic recovery (1). A potential strategy for the treatment of traumatic brain injury is transplantation of bone marrow stem cells (2-3). These cells appear to enter through the blood-brain barrier and selectively migrate to the traumatic hemisphere of the damaged brain to improve neurological recovery (2-3). However, because cell transplantation requires surgical intervention, it is clinically desirable to explore less invasive therapeutic procedures. Administration of granulocyte colony-stimulating factor (G-CSF) is known to mobilize hematopoietic stem cells (HSCs) from bone marrow into peripheral blood (4). Peripheral blood-derived HSCs have been used in place of bone marrow cells in transplantation for the regeneration of non-hematopoietic tissues such as skeletal muscle and heart (5). G-CSF has been used extensively for 10 years in the treatment of neutropenia, as well as for bone marrow reconstitution and stem cell mobilization (6). In traumatic brain

injury treatment through administration of stem cells (2), main determinants are critical for the colonization and trans-differentiation of stem cells into a variety of tissues: (1) traumatic tissue damage and (2) the number of circulating stem cells available (5). Under ischemic conditions, circulating stem cells appear to selectively migrate into ischemic regions to support plasticity and functional recovery of damaged tissue (5). Expression of stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 after focal cerebral ischemia (7) led us to speculate that this chemokine may also signal adhesion and migration of HSCs to ischemic tissue. On this basis, we hypothesized that traumatic brain injury enhances HSC plasticity and provides an environment that enhances differentiation of HSCs into original lineage cell types of the damaged organ such as astrocyte. In this study, we used a rat model to test the hypothesis that chemokines could mobilize HSCs in a manner similar to that in which they target inflammatory cells in non-neuronal damaged tissues. A sufficient number of HSCs mobilized by G-CSF, could then home in on cerebral traumatic injuries to promote neuronal repair and recovery of function; this would provide a basis for the development of a non-invasive autologous therapy for cerebral ischemia and traumatic brain injury. To date, no one has analyzed the effect of G-CSF on morphological and functional recovery after traumatic brain injury in rat.

2. Methods

2.1. Animal Model

A controlled cortical impact model in rat was used. Adult male Sprague-Dawley rats (weight: 250 to 300 g) were used in this study. Rats (n = 40) were anesthetized with chloral hydrate (350 mg/kg body weight) intraperitoneally. Rectal temperature was controlled at 37° ± 0.5°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Rats were placed in a stereotaxic frame. Meanwhile, 10-mm diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The contralateral craniotomy allowed lateral movement of cortical tissue. The dura was kept intact over the cortex. Injury was induced by impacting the left cortex (ipsilateral cortex) with a piston containing a 6-mm diameter tip at the rate of 4 m/s and 2.5 mm of compression. Animals were divided into three groups as follows: group 1 (10): TBI + saline (control), group 2 (10): TBI + Brdu (intraperitoneally), group 3 (10): TBI + G-CSF+ Brdu (intraperitoneally).

2.2. Bromodeoxyuridine Labeling

Bromodeoxyuridine (BrdU), a thymidine analogue that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling (Sigma chemical). The labeling protocol has been described previously (10). Pulse labeling was used to observe the time course of proliferative cells in the brain after cerebral ischemia. Experimental rats (including 10 G-CSF–treated rats and 10 control rats) were injected intraperitoneally with BrdU (50 mg/kg) every 24 hours for 14 consecutive days. Rats were killed 42 days after traumatic brain injury.

2.3. Experimental Animals and G-CSF Treatment

One day after induction of traumatic brain injury, rats were injected subcutaneously with recombinant human G-CSF (50 µg/kg) per day; once daily for 5 days (9). Vehicle animals were subjected to traumatic brain injury and injected with saline and Brdu.

2.4. Neurological Functional Evaluation

Neurological function in the rats was assessed using the neurological severity scores (NSS). The NSS is composed of motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), reflex, and beam walking tests. In the severity scores of injury, one point is awarded for the inability to correctly perform the tasks or for the lack of a tested reflex. The higher the NSS score is, the more severe will be the injury. The evaluation of all rats was started before TBI and performed after TBI at 1 week and weekly thereafter. All measurements were performed by observers blinded to individual treatment. The table 1 shows a set of modified neurological severity scores used to grade neurological function (30).

2.5. Immunohistochemistry of Brain Tissue

The brains of experimental rats were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde. The cerebral injured tissues (5 mm) were cut into coronal paraffin blocks. A series of 6-mm-thick sections at various levels (100-µm interval) were cut from this block and were analyzed by fluorescent microscopy For BrdU immunostaining. DNA was first denatured by incubating each section in 50% formamide of 2X standard saline citrate at 65 °C for 2 hours, then in 2 N HCl at 37 °C for 30 minutes, and finally rinsed in 0.1 mol/l boric acid with pH 8.5. Sections were then rinsed with Tris buffer and treated with 1% H2O2 to block endogenous peroxidase. After de-

anti-mouse IgG-rhodamine conjugated (dilution 1:60 in PBS (Chemicon)). Quantification of BrdU-immunoreactive cells was performed on paraffin-embedded tissue sections and was counted digitally with the use of a 40 objective lens via a computer imaging analysis system. Cerebral cells with uniform nuclear BrdU immunostaining were counted as previously described (12). All BrdU-reactive cells with BrdU were counted for all 10 coronal sections.

2.6. Statistical Analysis

Data were analyzed by ANOVA for multiple comparisons. In all experiments, means ± SD were calculated and represented. All data were analyzed using ANOVA for comparison between the groups with SPSS 15.0. A p value less than 0.05 was considered a statistically significant difference and $p<0.01$ was defined as a very significant difference. All p values >0.05 were interpreted as representing no significant difference.

3. Results

G-CSF stimulates stem cell mobilization and homing to brain after traumatic brain injury

BrdU-reactive cells detected from an average of 10 histology slides per treatment animal from multiple areas of the ipsilateral hemisphere including cortices and striatum of the ipsilateral hemisphere. The vast majority of BrdU-labeled cells were located in the traumatic core and its boundary zone. Few cells were observed in the contralateral hemisphere. In summary, G-CSF–treated traumatic rats exhibited significantly increased numbers of BrdU immunoreactive cells in their traumatic core compared with saline- and Brdu only injected traumatic rats (Figures 1-3).

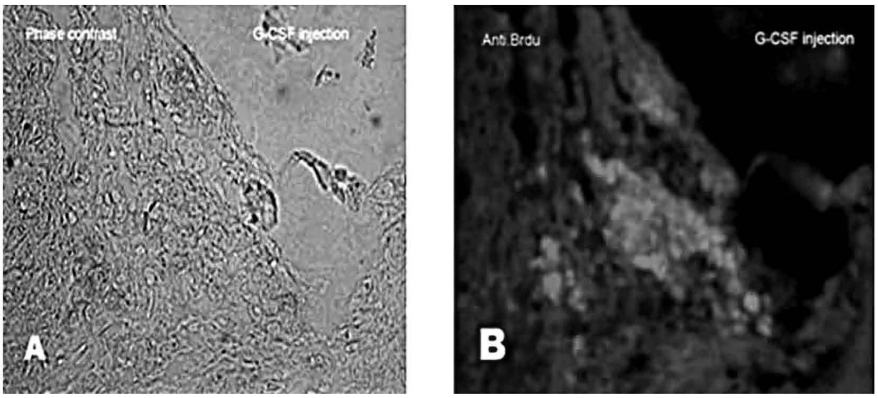

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Figure 1. Immunofluorescent staining fluorescent microscopy (G-CSF treatment group). BrdU-immunoreactive cells (B, red) in traumatic region of rat brains

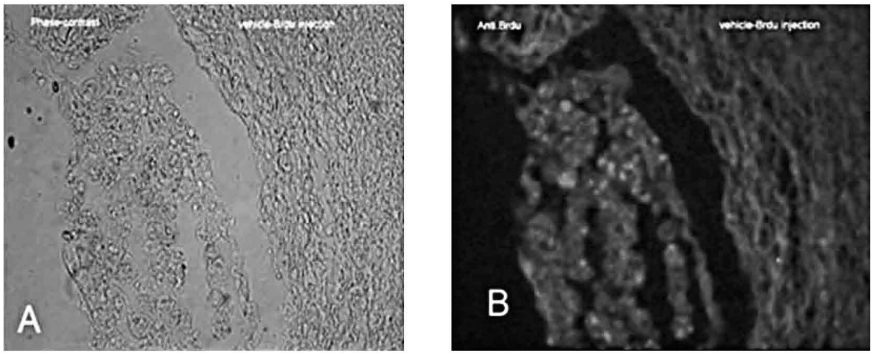

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Figure 2. Immunofluorescent staining fluorescent microscopy (only Brdu injected group). BrdU-immunoreactive cells (B, red)

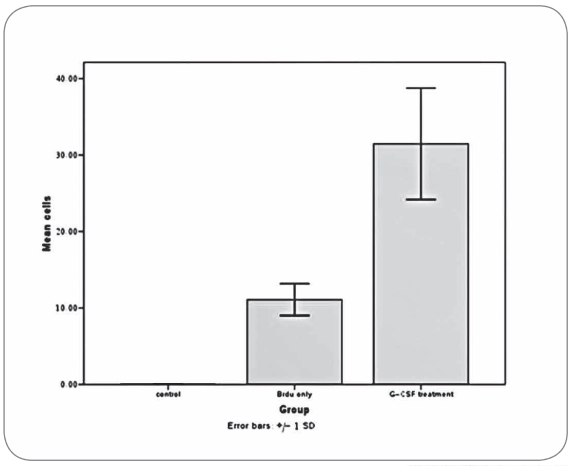

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Figure 3. Brdu cell counting (ANOVA analysis) Neurological and motor function evaluation

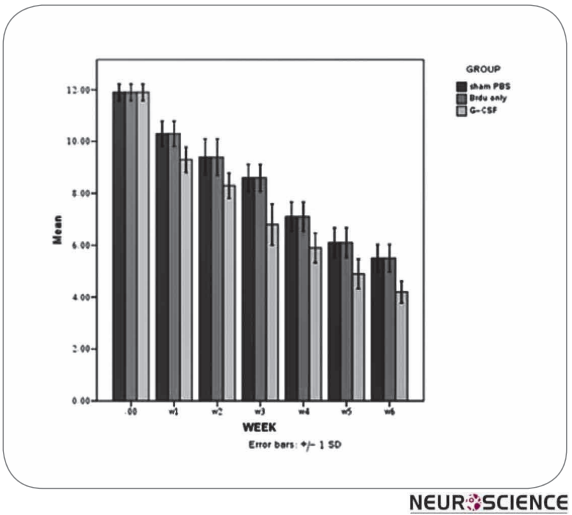

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Figure 4. Results of behavioral functional tests (modified neurological severity score [mNSS] test) before and after traumatic brain injury (TBI). Rats were injured to traumatic brain injury alone (n=10) or were injected with G-CSF (n=10) or vehicle (Brdu only) (n=10) 1 day after TBI. Significant functional recovery was detected in rats treated with G-CSF compared with control and vehicle. P value<0.01

Table 1. Modified neurological severity scores used to grade neurological function

Modified neurological severity score (mNss)	
Motor tosts	**Points**
Raising the rat by the tail	**3**
1 Flexion of forelimb	
1 Flexion of hindlimb	
1 Head moved more than 10° to the vertical axis	
Walking on the floor (normal=0; maximum=3)	3
0 Normal walk	
1 Inability to walk straight	
2 Circlling toward the parectic side	
3 Falling down to the parectic side	
Sensory test	**2**
1 Placing test (visual and tactile test)	
2 Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	
Beam balance tests (normal=0; maximum=6)	**6**
0 Balances with steady posture	
1 Grasps side of beam	
2 Hugs the beam and one limb falls down from the beam	
3 Hugs the beam and two limbs fall down from the beam, or spins on beam (>60 s)	
4 Attempts to balance on the beam but falls off (>40 s)	
5 Attempts to balance on the beam but falls off (>20 s)	
6 Falls off: no attempt to balance or hang on to the beam (<20 s)	
Reflexes absent and abnormal movements	**4**
1 Pinna reflex (a nead shake when the auditory meatus is touched)	
1 Corneal reflex (an eye blink when the cornea is lightly touched with cotton)	
1 Startle reflex (a motor response to a brief noise from snapping a clipboard and paper)	
1 Seizures, myoclonus, myodystony)	
Maximum points	**18**
One point is awarded for the inability to perform the task or for the lack of a tested reflex: 13-18- severe injury; 7-12- moderate injury; 1-6- mild injury.	

4. Discussion

In this study, we demonstrated that subcutaneous injections of G-CSF, starting 1 day after traumatic brain injury and continuing for up to 5 days, enhance functional repair in rats suffering from traumatic injury with significant recovery of neurological dysfunction. It is likely that the mechanisms providing therapeutic benefit in this study are multidimensional. First, it found that administration of G-CSF increased the mobilization of circulating HSCs to damaged areas of the brain. Second, it is possible that interaction of HSCs with traumatic tissue may lead HSCs and/or parenchymal cells to produce trophic factors (13) that may contribute to the recovery of neural functions

lost as a result of tissue injury (14). HSCs have been shown to constitutively express interleukins such as interleukin (IL-1), IL-8, and IL-16, fibroblast growth factor-2, vascular endothelial growth factor, insulin growth factor-1, granulocyte-monocyte colony-stimulating factor, and tumor necrosis factor-α (15). These cytokines may act as survival, growth, and/or differentiation factors for neuronal and vascular progenitor cells, which may in turn proliferate, migrate, and differentiate after brain injury and thus contribute to damage recovery processes. Neurotrophic factors have been shown to enhance neuronal sprouting (16), synaptogenesis (17), and neurotransmission (18) and increase neurotrans-

mitter release (19). In the case of the glial cell line–derived neurotrophic factor, its injection into the brain was found to greatly diminish infarction volume and improve neurological functions in rats suffering cerebral ischemia (20). Therefore, some G-CSF–mobilized HSCs could enter the cerebral injury region and interact with brain cells; this interaction may enhance the production of trophic factors such as glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor, which may in turn promote repair (3) of damaged parenchymal cells after traumatic brain injury in rats. In this study, more Brdu labeled cells were found in the traumatic hemisphere than in the vehicle of experimental rats. This suggests that disruption of the blood-brain barrier may facilitate selective entry of HSCs into the traumatic rather than the non-traumatic contralateral hemisphere. It is interesting to note that in traumatic and ischemic rat brain a number of neurotrophic factors are released, which have been shown to result in human bone marrow stromal cell growth factor production (3, 21). Therefore, we speculate that traumatic damage to brain tissue may result in the release of trophic factors, which in turn may target HSCs to damaged tissues. Similarly, Chen and colleagues (29) reported that intravenous administration of marrow stromal cells in rats results in their accumulation in the ischemic brain, and, in a model of hepatic injury, regenerated hepatic cells were shown to be of bone marrow origin (29). These findings suggest that the "injured" brain might specifically attract bone marrow–derived cells. It will be important to clarify which signaling molecules attract HSCs and direct their migration to damaged areas. A recent report (23) has indicated that SDF-1 is a strong chemoattractant for CD34 cells that express CXCR4, the receptor for SDF-1, and plays an important role in HSC trafficking between peripheral circulation and bone marrow. Recently, Stumm et al (7) demonstrated that focal cerebral ischemia causes an increase in SDF-1 expression in regions adjacent to the infarcted area. Lataillade and colleagues (24) reported that a significant proportion of HSCs, mobilized by G-CSF, express CXCR4 receptors on their cell surface and that SDF-1 induces directional migration of HSCs. By attracting HSCs to the ischemic region, an SDF-1/CXCR4 interaction may be directly involved in vascular remodeling, angiogenesis, and neurogenesis, thereby alleviating stroke symptoms. In addition, HSCs migrating to the ischemic hemisphere could create local chemical gradients and/or localized chemokine accumulation, dictating a directional response in endothelial, neuronal, and glial progenitor cells (26). As a consequence of this autocrine regulatory pathway, endothelial and neuronal progenitor cells could mobilize and fuse with each other, a step required for subsequent formation of a structured network of branching vessels and neurons (27). In addition to mobilized HSCs, SDF-1 might also stimulate host endothelial progenitor cell differentiation from preexisting blood vessels and/or host endothelial progenitor cells derived from bone marrow (27). In addition to inducing HSC migration to ischemic regions, SDF-1 has also been shown to exert survival effects on cultured CD34 cells (26) and to regulate endothelial cell branching morphogenesis (28). Taken together, we therefore hypothesize that plasma levels of SDF-1, released from damaged tissues, may provide a host defense signal that in turn attracts mobilizing HSCs to repair the disordered tissue. This study also provides evidence that the ultimate degree of neurological improvement is dependent on the recruitment of sufficient HSCs to the damaged area of brain at an early stage after tissue injury. We propose that the G-CSF treatment may mobilize autologous HSCs into circulation, enhance their translocation into traumatic brain, and thus significantly improve lesion repair. We believe that the G-CSF therapeutic protocol reported here represents an attractive strategy for the development of a clinically significant noninvasive traumatic brain injury therapy.

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